CCAAT/enhancer-binding protein β is required for activation of genes for ornithine cycle enzymes by glucocorticoids and glucagon in primary-cultured hepatocytes

Tatsuya Kimura[a,b], Shoaib Chowdhury[a], Takashi Tanaka[c], Atsuko Shimizu[d], Katsuro Iwase[a,d], Seiichi Oyadomari[a], Tomomi Gotoh[a], Hiromitsu Matsuzaki[b], Masataka Mori[a], Shizuo Akira[c], Masaki Takiguchi[a,d,*]

[a] *Department of Molecular Genetics, Kumamoto University School of Medicine, Kumamoto 860-0811, Japan*
[b] *Department of Internal Medicine, Kumamoto University School of Medicine, Kumamoto 860-0811, Japan*
[c] *Research Institute for Microbial Diseases, Osaka University, Suita 565-0871, Japan*
[d] *Department of Biochemistry, Chiba University School of Medicine, Inohana 1-8-1, Chiba 260-8670, Japan*

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Abstract Transcription of genes for enzymes of the ornithine cycle is activated by hormones such as glucocorticoids and glucagon. Promoters and enhancers of several genes for the enzymes interact with the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors, and C/EBPβ has been suggested to mediate glucocorticoid response of the gene for arginase, the last enzyme of the cycle. To determine the contribution of C/EBPβ to hormonal regulation of genes for ornithine cycle enzymes, we examined mice with targeted disruption of the C/EBPβ gene. Induction of genes for the enzymes by intraperitoneal injection of dexamethasone and glucagon was almost intact in the liver of C/EBPβ-deficient mice. On the other hand, in primary-cultured hepatocytes derived from C/EBPβ-deficient mice, induction of genes for the first enzyme carbamylphosphate synthetase, as well as for arginase, in response to dexamethasone and/or glucagon was severely impaired. Therefore, C/EBPβ is required for hormonal induction of the genes for ornithine cycle enzymes in primary-cultured hepatocytes, while the deficiency of C/EBPβ is compensated for in vivo.

Key words: Transcription factor; Gene regulation; Hormone; Dexamethasone; Gene targeting; Knockout mouse

1. Introduction

The ornithine cycle in the liver is an enzyme system which converts toxic ammonia derived from amino acids into urea. The cycle consists of five enzymes, i.e. carbamylphosphate synthetase (CPS), ornithine transcarbamylase (OTC), argininosuccinate synthetase (AS), argininosuccinate lyase (AL), and arginase. Genes for the enzymes are induced during perinatal period largely in a coordinated manner, and postnatally are regulated in response to dietary changes (for review see [1,2]). These regulations of the genes are mediated mainly by three hormones: glucagon and glucocorticoids induce, while insulin represses, the genes for ornithine cycle enzymes except for OTC [1,2].

Induction of mRNAs for the enzymes by glucagon via the cAMP pathway is known as the primary hormonal response, since it follows a rapid time course without a lag time, and since it does not require on-going protein synthesis [3]. A transcription factor(s) that acts as the target of the cAMP pathway and is responsible for the induction remains to be identified. On the other hand, the induction by glucocorticoids follows a delayed time course with a lag time of several hours and is blocked by a protein synthesis inhibitor cycloheximide, exhibiting the typical feature of the secondary hormonal response that is mediated by a newly synthesized transcription factor(s) [3].

The CCAAT/enhancer-binding protein (C/EBP) family is the prototype family of basic region leucine zipper (bZIP)-type transcription factors [4,5] (for review see [6]). We have postulated that C/EBPβ, a member of this family [7–12], mediates the secondary glucocorticoid response [13,14]: glucocorticoids stimulate transcription of the C/EBPβ gene [13], and newly synthesized C/EBPβ in turn activates the arginase gene through binding to the delayed glucocorticoid-responsive element of the arginase enhancer [14]. It has been also suggested that C/EBPβ is involved in glucagon response, based on the observations that the C/EBPβ gene is induced by glucagon/cAMP [13,15,16], and that C/EBPβ is phosphorylated by stimulation of the cAMP/protein kinase A pathway [17–19].

Besides the arginase enhancer, C/EBP family members interact with the promoter and enhancer regions of CPS [20,21], the enhancer region of OTC [22,23], and the promoter region of arginase [24,25]. Previously we showed that neonatal mice lacking C/EBPα [26] suffer from hyperammonemia associated with impaired expression of the genes for ornithine cycle enzymes [27], demonstrating a pivotal role of C/EBPα in expression of the genes for ornithine cycle enzymes during perinatal period. Here we examined whether disruption of the C/EBβ gene [28,29] affects expression of genes for the enzymes. In

*Corresponding author. Fax: (81)-43-226 2037.
E-mail: mtak@med.m.chiba-u.ac.jp

Abbreviations: CPS, carbamylphosphate synthetase; OTC, ornithine transcarbamylase; AS, argininosuccinate synthetase; AL, argininosuccinate lyase; C/EBP, CCAAT/enhancer-binding protein; MT, metallothionein; TAT, tyrosine aminotransferase; PEPCK, phosphoenolpyruvate carboxykinase; GR, glucocorticoid receptor

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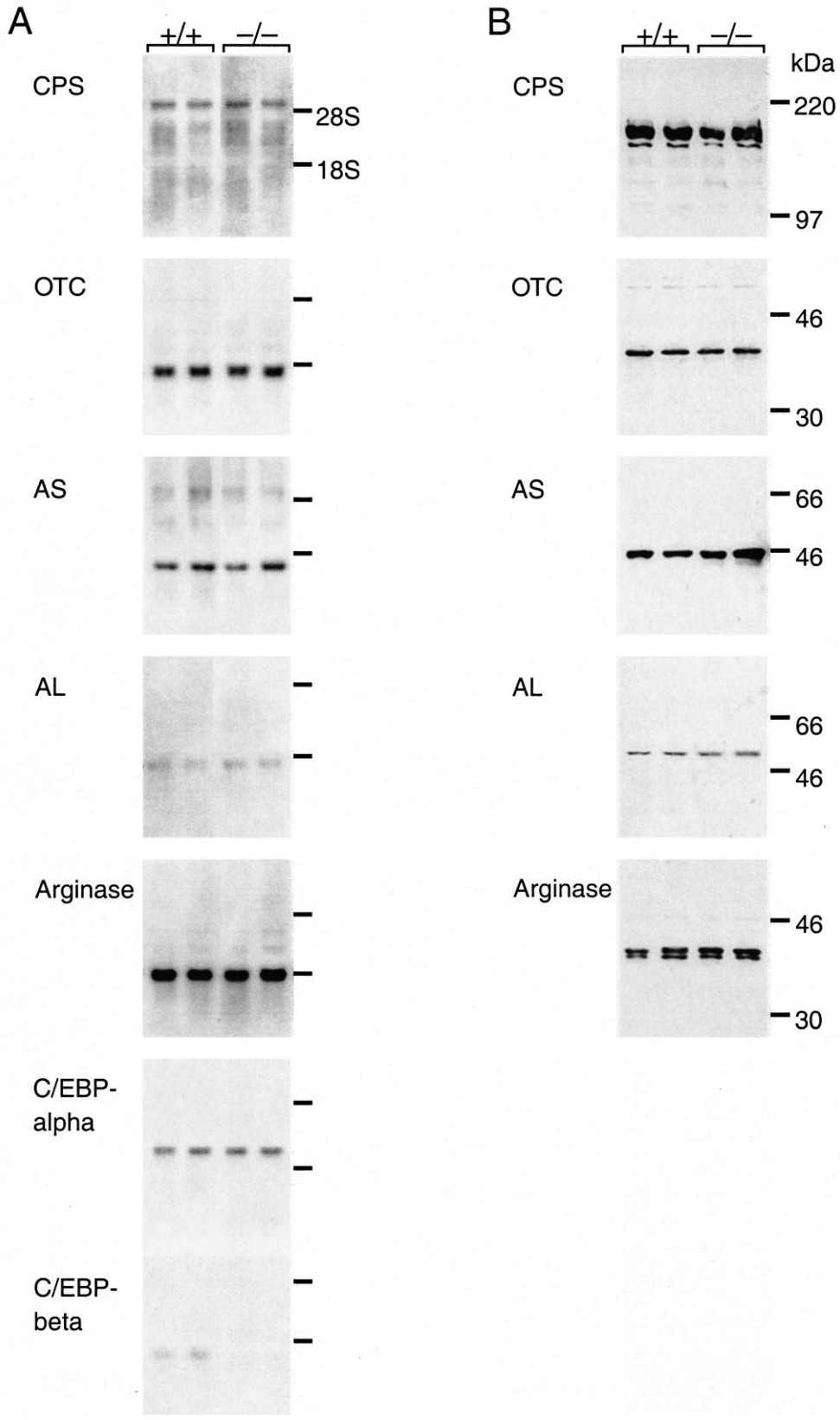

Fig. 1. Basal mRNA (A) and protein (B) levels for the ornithine cycle enzymes in livers of C/EBPβ-deficient mice. Total RNAs and tissue extracts were prepared from the livers of wild-type (+/+) and C/EBPβ-deficient (−/−) mice fed on standard diet, and subjected to RNA blot (A) and immunoblot (B) analysis, respectively, as described in Section 2. Positions of 28S and 18S rRNAs (A) and molecular weight markers (rainbow markers, Amersham) consisting of myosin (220 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (46 kDa) and carbonic anhydrase (30 kDa) (B) are indicated.

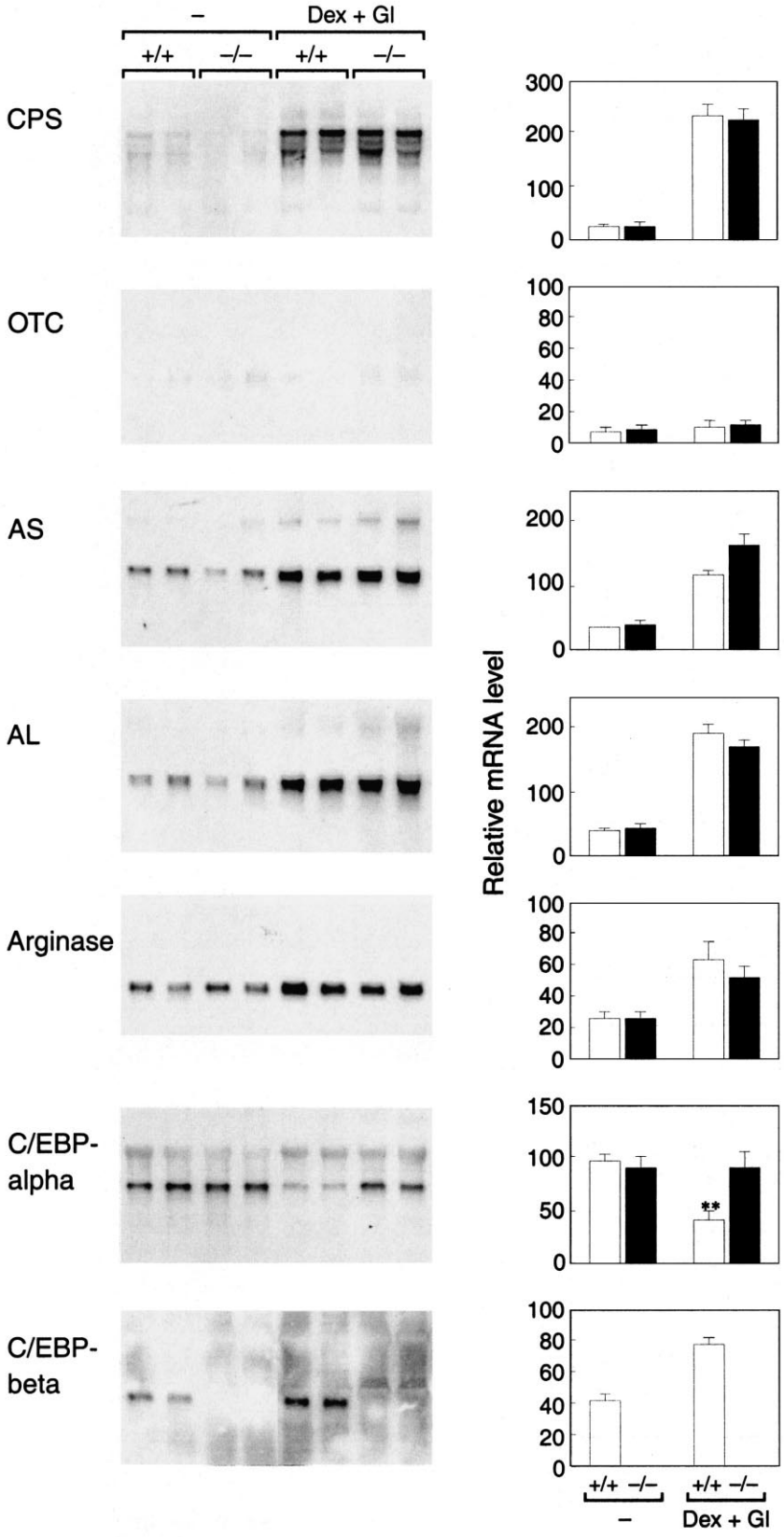

Fig. 2. Intactness of hormonal regulation of mRNA levels for ornithine cycle enzymes in livers of C/EBPβ-deficient mice. Wild-type (+/+) and C/EBPβ-deficient (−/−) mice, which had been fed on protein-free diet to decrease basal mRNA levels for ornithine cycle enzymes, were treated with a combination of dexamethasone and glucagon (Dex+Gl), or their vehicles (−) as described in Section 2. Total RNAs derived from the livers of mice were subjected to RNA blot analysis. Representative chemiluminograms are shown on the left. Quantified results of the chemiluminograms are presented on the right. mRNA levels relative to the level (100%) for wild-type mice on standard diet (Fig. 1) were assessed for three independent experiments, and are represented as the mean+S.E.M. Significant difference was evaluated by Student's *t*-test. **, $P < 0.01$ vs. other groups.

primary-cultured hepatocytes of the C/EBPβ-deficient mice, induction of genes for CPS and arginase by dexamethasone and/or glucagon was severely impaired, indicating that C/EBPβ is essential in hormonal response of the genes for ornithine cycle enzymes in primary hepatocytes.

2. Materials and methods

2.1. Animals

Mice heterozygous for the disruption of the C/EBPβ gene [28] were mated, and genotypes of their offspring were determined by Southern blotting as described [28].

2.2. Diet and hormone administration

Following a shift to feeding with protein-free diet (Clea Japan Inc., Tokyo, Japan) on day 1, mice were injected intraperitoneally with dexamethasone (5 μg/g of body weight) and glucagon (2 μg/g of body weight) twice at 21:00 on day 6 and at 9:00 on day 7, and were killed at 13:00.

2.3. Primary culture of hepatocytes and treatment with hormones

Mice fed on standard diet (25% protein; Clea) were subjected to isolation of parenchymal hepatocytes by in situ collagenase perfusion as described [30,31] on day 1, and 3×10^6 hepatocytes were plated onto collagen-coated plastic dishes (100 mm diameter; Corning Inc., Corning, NY, USA) with 10 ml of Dulbecco's modified Eagle's medium containing 5% fetal calf serum and 100 μg/ml of streptomycin. The medium was changed 24 h after the plating. On day 3, cells were treated with 1×10^{-6} M dexamethasone, 3×10^{-8} M glucagon, or a combination of both hormones for 4 or 12 h.

2.4. RNA blot analysis

Total RNA was isolated from the liver or cultured hepatocytes using the acid guanidinium thiocyanate-phenol-chloroform extraction procedure [32]. RNA (2 μg per lane) was electrophoresed in denaturing formaldehyde-agarose (1%) gels. After visualizing 28S and 18S rRNAs by ethidium bromide staining to check the integrity of RNA samples and equal loading, the RNA was blotted onto nylon membranes. Digoxigenin-labeled antisense RNA probes were synthesized using a transcription kit (Roche Diagnostics, Tokyo, Japan), from cDNAs for the following proteins: CPS at nucleotide positions 2799–3237 [33], OTC [34], AS at nucleotide positions 326–775 [35], AL [36], arginase [37], C/EBPα [23], C/EBPβ [23], metallothionein (MT) [38], and tyrosine aminotransferase (TAT) [39]. Hybridization, washing and chemiluminescent detection on X-ray films were done as recommended by Roche Diagnostics. Densitometric quantification was done using MacBas software (Fuji Photo Film Co., Tokyo, Japan).

2.5. Immunoblot analysis

Mouse livers were homogenized in nine volumes of 20 mM potassium HEPES buffer (pH 7.4) containing 0.5% Triton X-100, 1 mM dithiothreitol, 50 μM antipain, 50 μM leupeptin, 50 μM chymostatin, and 50 μM pepstatin. The homogenates were centrifuged at $25\,000\times g$ for 30 min at 4°C, and the supernatants were used as tissue extracts. The extracts (20 μg of protein) were subjected to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis, and proteins were electrotransferred to nitrocellulose membranes. Immunodetection was performed using an ECL kit (Amersham Pharmacia Biotech, Tokyo, Japan) and antibodies against CPS [40], OTC [41], AS [42], AL [42] and arginase [43]. Rainbow markers (Amersham Pharmacia Biotech) were employed as molecular weight standards.

3. Results

3.1. Expression of genes for ornithine cycle enzymes in the liver of C/EBPβ-deficient mice

We previously showed that expression of genes for ornithine cycle enzymes is impaired in the liver of C/EBPα-deficient mice [27]. Here we examined whether it is also the case for C/EBPβ-deficient mice. Livers of 10- to 16-week-old C/EBPβ$^{-/-}$ mice fed on standard diet were subjected to RNA blot and immunoblot analyses. As shown in Fig. 1, mRNA and protein levels for ornithine cycle enzymes were almost comparable between C/EBPβ$^{-/-}$ and control C/EBPβ$^{+/+}$ mice. Therefore, basal expression of the enzyme genes is intact in C/EBPβ-deficient mice.

Hepatic C/EBPβ is induced by glucocorticoids and glucagon [13], and is likely to be involved in induction of the arginase gene, especially in response to glucocorticoids [14]. We investigated whether induction of the genes for ornithine cycle enzymes by the hormones is impaired or not in C/EBPβ-deficient mice (Fig. 2). Prior to administration of the hormones, mice were fed on protein-free diet for 5 days, to reduce basal mRNA levels for the enzymes and to see stronger fold induction by the hormones, as described previously [44]. Subsequently, mice were injected with a combination of dexamethasone and glucagon intraperitoneally. As shown in Fig. 2, mRNA levels for the ornithine cycle enzymes, except for OTC, were raised by the hormones, and their extents were similar between C/EBPβ$^{-/-}$ and C/EBPβ$^{+/+}$ mice. Therefore, hormonal induction of the genes for the enzymes is intact in C/EBPβ$^{-/-}$ mice in vivo.

C/EBPβ mRNA levels in C/EBPβ$^{+/+}$ mice were increased in response to the hormones (Fig. 2), concordant with our previous results obtained with primary-cultured rat hepatocytes [13]. Interestingly, the hormonal treatment lowered C/EBPα mRNA levels by 56% in C/EBPβ$^{+/+}$ mice, but not in C/EBPβ$^{-/-}$ mice. The escape from the hormonal repression of the C/EBPα gene in C/EBPβ$^{-/-}$ mice might be a compensatory change for lack of C/EBPβ.

3.2. Impaired hormonal induction of mRNAs for CPS and arginase in primary-cultured C/EBPβ-deficient hepatocytes

To examine immediate effects of hormones on hepatocytes, we isolated and cultured primary hepatocytes from C/EBPβ$^{-/-}$ and wild-type mice. The hepatocytes were treated with dexamethasone and/or glucagon for 4 or 12 h, and RNA blot analysis was carried out (Fig. 3). In wild-type hepatocytes, mRNA levels for CPS were increased by each hormone and further by their combination. In C/EBPβ$^{-/-}$ hepatocytes, these increases were almost completely lost. OTC mRNA levels showed no apparent change in response to the hormones in both genotypes. Increases in mRNA levels for AS and AL were apparent with a combination of the hormones in wild-type hepatocytes, and seemed to be marginally impaired in

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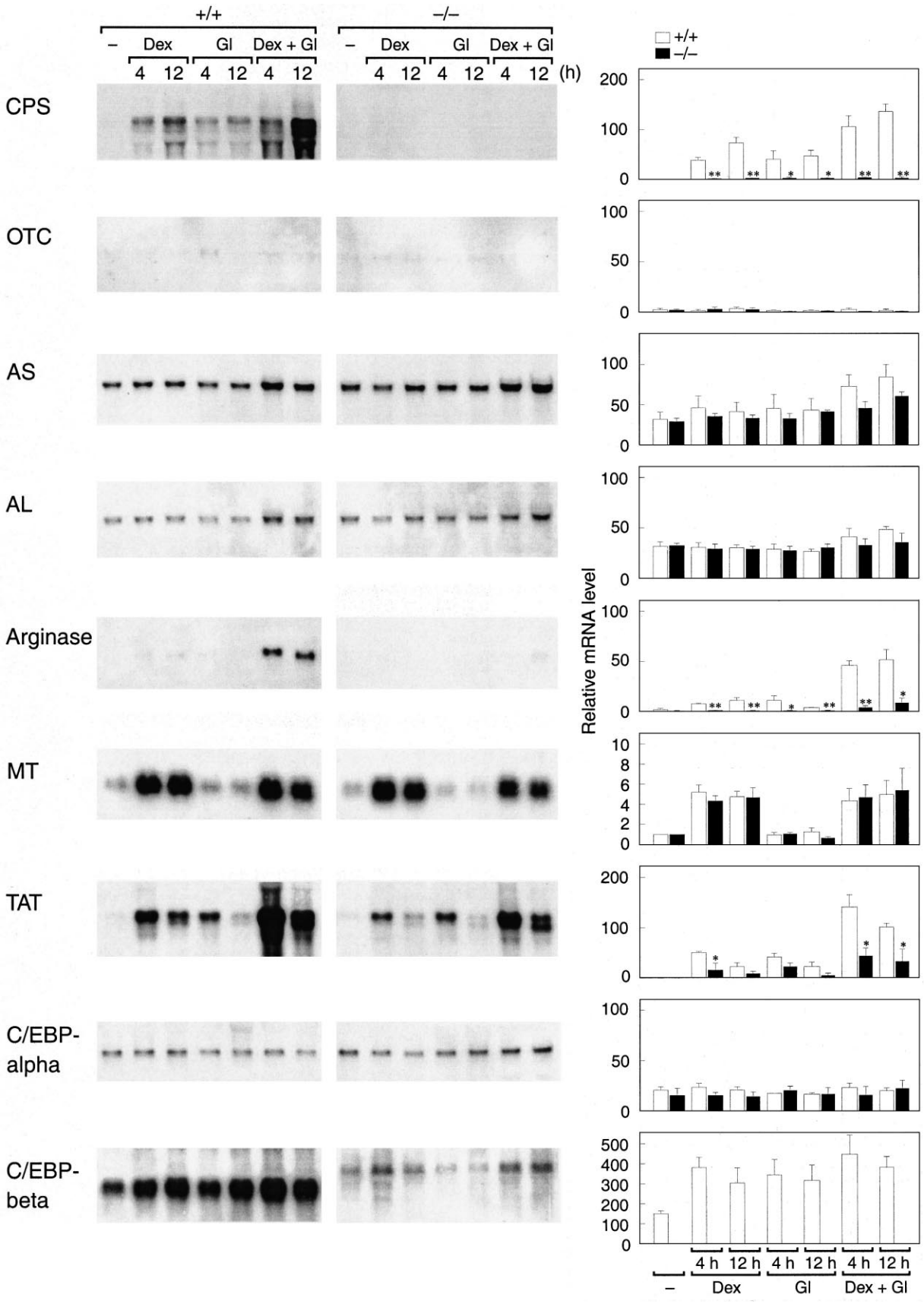
Fig. 3. Impairment of hormonal regulation of mRNA levels for ornithine cycle enzymes in primary-cultured hepatocytes of C/EBPβ-deficient mice. Hepatocytes derived from wild-type (+/+) and C/EBPβ-deficient (−/−) mice were treated with 1×10^{-6} M dexamethasone (Dex), 3×10^{-8} M glucagon (Gl) or a combination of both hormones (Dex+Gl), or not treated (−), for 4 or 12 h. Total RNAs prepared from the hepatocytes were subjected to RNA blot analysis. Representative chemiluminograms are shown on the left. Quantified results of the chemiluminograms are presented on the right. mRNA levels are expressed as values relative to the level (100%) in the liver of wild-type mice on standard diet (Fig. 1), except MT mRNA for which the level in cultured hepatocytes without hormonal treatment is assigned the value of one because of fluctuation of MT mRNA levels in the liver of alive mice. Data were assessed for at least three independent experiments, and are represented as the mean+S.E.M. Significant difference from wild-type (+/+) mice was determined by Student's *t*-test: **, $P<0.01$; *, $P<0.05$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C/EBPβ$^{-/-}$ cells. mRNA levels for arginase in wild-type hepatocytes were increased moderately by each hormone, and strongly by their combination. In C/EBPβ$^{-/-}$ hepatocytes, these increases were severely impaired.

As positive controls for hormonal responses, expression of genes for MT and TAT was examined (Fig. 3). Induction of the MT genes by glucocorticoids [45,46] and induction of the TAT gene by glucocorticoids [47,48] or glucagon/cAMP [49] have been reported to be caused in a primary manner. mRNA levels for MT were raised by dexamethasone to the same extent in C/EBPβ$^{-/-}$ and control hepatocytes. Induction of the TAT gene by dexamethasone was significantly but only partially impaired in C/EBPβ$^{-/-}$ hepatocytes compared to wild-type cells. Glucagon induction of the TAT gene was also marginally impaired in C/EBPβ$^{-/-}$ hepatocytes. Therefore, while C/EBPβ is required for full hormonal induction of the TAT gene, basic signal transduction pathways for the hormones are almost intact in C/EBPβ$^{-/-}$ mice. Taken together with intactness of glucocorticoid induction of the MT genes, these observations serve as controls for specific impairment of hormonal induction of the genes for ornithine cycle enzymes, especially for CPS and arginase, in C/EBPβ$^{-/-}$ hepatocytes.

Increases in the C/EBPβ mRNA levels by dexamethasone and/or glucagon were apparent in wild-type hepatocytes (Fig. 3), concordant with in vivo results described above and our previous results for rat hepatocytes [13]. No significant variation in mRNA levels for C/EBPα was observed by any hormonal treatment in wild-type as well as C/EBPβ$^{-/-}$ hepatocytes, showing no apparent compensatory change in expression of the C/EBPα gene. The cause for discrepancy in hormonal response of the C/EBPα gene between in vivo and in cultured hepatocytes remains to be investigated.

4. Discussion

In this study, we demonstrated that increases in mRNA levels for the ornithine cycle enzymes, especially for CPS and arginase, in response to dexamethasone and/or glucagon were severely impaired in primary-cultured hepatocytes derived from C/EBPβ$^{-/-}$ mice. Most likely, C/EBPβ is essential for hormonal induction of the enzyme genes at transcription levels. On the other hand, in vivo, the lack of C/EBPβ seems to be compensated for, since no apparent defect of the hormonal induction was detected in C/EBPβ$^{-/-}$ mice. A possible mechanism for this compensation is that C/EBPα escapes from hormonal downregulation and persists at sufficient levels in the liver of C/EBPβ-deficient mice. However, many investigations remain to be done to examine this hypothesis. Molecular mechanisms for the hormonal repression of the C/EBPα gene and for its impairment in C/EBPβ-deficiency also remain to be determined.

Contribution of C/EBPβ to glucocorticoid response in both primary and secondary manners has been suggested. The primary glucocorticoid response, which is triggered by the hormone–nuclear receptor complex, sometimes requires cooperation with other transcription factors. C/EBPβ is such an accessory factor involved in the glucocorticoid induction of the gene for phosphoenolpyruvate carboxykinase (PEPCK) [50]. The glucocorticoid receptor (GR) and C/EBPβ also synergistically activate the α_1-acid glycoprotein gene in acute-phase response [51–53] presumably by direct protein–protein interaction between these two factors [52] and by sharing a coactivator [53]. Functional synergism between the GR and C/EBPβ in a manner that does not depend on direct protein–protein interaction nor on DNA-binding of the receptor was also reported [54]. As for the secondary glucocorticoid response, expression of the C/EBPβ gene itself is induced by glucocorticoids in a primary manner [13], and newly synthesized C/EBPβ in turn activates target genes such as the arginase gene [14]. Therefore, deficiency of C/EBPβ potentially can result in impairment of both primary and secondary glucocorticoid responses.

Since induction of the arginase gene by glucocorticoids is caused mainly in a secondary manner [3], impairment of the induction in C/EBPβ-deficient mice is attributable to lack of newly synthesized C/EBPβ. Glucocorticoid response of the CPS gene was at first noted to be secondary, based on delayed time course and sensitivity to an inhibitor of protein synthesis [3]. On the other hand, recent studies [21] showed that the glucocorticoid response unit of the CPS enhancer interacts directly with the GR as well as C/EBP family members, suggesting involvement of the GR also in a primary manner and its modification by C/EBP family members. Their exact roles in the primary and secondary glucocorticoid response remain to be clarified.

Complicated glucocorticoid regulation of the CPS gene resembles that of the PEPCK gene. The promoter region of the PEPCK gene also interacts with the GR and C/EBP family members [50,55], while a report [56] described that glucocorticoid response of this gene is caused in a secondary manner. Recently, it was briefly noted that glucocorticoid induction of the PEPCK gene was impaired in the liver of C/EBPβ$^{-/-}$ mice [57].

Glucocorticoid induction of the TAT gene [47,48] as well as of the MT genes [45,46] is directly triggered by the GR/hormone complex in a primary manner. In this study, dexamethasone induction of the TAT gene, but not of the MT genes, was partially impaired in primary hepatocytes of C/EBPβ$^{-/-}$ mice. It has been reported that induction of the TAT gene by the GR/hormone complex is synergistically stimulated by HNF-3 family members [58,59], and potentially also by C/EBP family members [59]. The partial impairment of glucocorticoid induction of the TAT gene in C/EBPβ$^{-/-}$ mice might have resulted from loss of this synergism with C/EBPβ.

In the previous studies, contributions of C/EBPα and C/EBPβ to transcriptional regulation by glucagon/cAMP were controversial. While mRNA and protein levels of C/EBPβ in the liver or cultured hepatocytes are upregulated by glucagon/cAMP [13,15,16], those of C/EBPα have been reported to be unchanged [13,16] or rather decreased [15]. In addition, C/EBPβ, but not C/EBPα, has been shown to be phosphorylated by stimulation of the cAMP/protein kinase A pathway [17–19]. Based on these observations, it was speculated that C/EBPβ is involved in mediating the effects of glucagon/cAMP more profoundly than C/EBPα is. However, recent studies with knockout mice [16] or antisense RNA expressed in hepatoma cells [60] suggested that C/EBPα, rather than C/EBPβ, is required for cAMP induction of the PEPCK gene, while C/EBPβ may compensate for the lack of C/EBPα when appropriately induced [16]. Since the present study suggests that C/EBPβ-deficiency might be compensated for by C/EBPα when present at sufficient levels, it is tempting to speculate that C/EBPα and C/EBPβ are reciprocally compensatory at least in part for their functions. Molecular mech-

anisms for induction of the CPS and arginase genes by glucagon/cAMP via C/EBPα and C/EBPα remain to be elucidated. It would be interesting to examine if binding sites for C/EBP family members in regulatory regions of genes for CPS [20,21] and arginase [14,24,61] can mediate effects of glucagon/cAMP.

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